RNA isolation

User manual

NucleoSpin® RNA
NucleoSpin® RNA Midi

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MACHEREY-NAGEL
www.mn-net.com

RNA isolation

Protocol-at-a-glance (Rev. 17)

		Mini	Midi
		NucleoSpin® RNA	**NucleoSpin® RNA Midi**
1	**Homogenize sample**	30 mg	100 mg
2	**Lyse cells**	350 µL RA1 3.5 µL ß-mercaptoethanol Mix	1.8 mL RA1 18 µL ß-mercaptoethanol Mix
3	**Filtrate lysate**	11,000 x *g*, 1 min	4,500 x *g*, 10 min
4	**Adjust RNA binding conditions**	350 µL 70 % ethanol Mix	1.8 mL 70 % ethanol Mix
5	**Bind RNA**	Load sample 11,000 x *g*, 30 s	Load sample 4,500 x *g*, 3 min
6	**Desalt silica membrane**	350 µL MDB 11,000 x *g*, 1 min	2.2 mL MDB 4,500 x *g*, 3 min
7	**Digest DNA**	95 µL DNase reaction mixture RT, 15 min	250 µL DNase reaction mixture RT, 15 min
8	**Wash and dry silica membrane**	1st wash 200 µL RAW2 2nd wash 600 µL RA3 3rd wash 250 µL RA3 1st and 2nd 11,000 x *g*, 30 s 3rd 11,000 x *g*, 2 min	1st wash 2.6 mL RAW2 2nd wash 2.6 mL RA3 3rd wash 2.6 mL RA3 1st and 2nd 4,500 x *g*, 3 min 3rd 4,500 x *g*, 5 min
9	**Elute highly pure RNA**	60 µL RNase-free H_2O 11,000 x *g*, 1 min	500 µL RNase-free H_2O RT, 2 min 4,500 x *g*, 3 min

MACHEREY-NAGEL GmbH & Co. KG · Neumann-Neander-Str. 6–8 · 52355 Düren · Germany
Tel.: +49 24 21 969-270 · Fax: +49 24 21 969-199 · tech-bio@mn-net.com · ***www.mn-net.com***

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1 Components

1.1 Kit contents

	NucleoSpin® RNA		
REF	**10 preps 740955.10**	**50 preps 740955.50**	**250 preps 740955.250**
Lysis Buffer RA1	10 mL	25 mL	125 mL
Wash Buffer RAW2	13 mL	13 mL	80 mL
Wash Buffer RA3 (Concentrate)*	6 mL	12 mL	3 x 25 mL
Membrane Desalting Buffer MDB	10 mL	25 mL	125 mL
Reaction Buffer for rDNase	7 mL	7 mL	30 mL
rDNase, RNase-free (lyophilized)*	1 vial (size D)	1 vial (size F)	5 vials (size F)
RNase-free H_2O	13 mL	13 mL	60 mL
NucleoSpin® Filters (violet rings)	10	50	250
NucleoSpin® RNA Columns (light blue rings – plus Collection Tubes)	10	50	250
Collection Tubes (2 mL)	30	150	750
Collection Tubes (1.5 mL)	10	50	250
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* For preparing of workings solutions and storage conditions see section 3.

Kit contents *continued*

	NucleoSpin® RNA Midi
REF	**20 preps 740962.20**
Lysis Buffer RA1	125 mL
Wash Buffer RAW2	80 mL
Wash Buffer RA3 (Concentrate)*	25 mL
Membrane Desalting Buffer MDB	50 mL
Reaction Buffer for rDNase	7 mL
rDNase, RNase-free (lyophilized)*	1 vial (size D)
RNase-free H_2O	13 mL
NucleoSpin® Filters Midi (plus Collection Tubes)	20
NucleoSpin® RNA Midi Columns (plus Collection Tubes)	20
Collection Tubes (15 mL)	20
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* For preparing of workings solutions and storage conditions see section 3.

1.2 Reagents, consumables, and equipment to be supplied by user

Reagents

- 96–100 % ethanol (to prepare Wash Buffer RA3)
- 70 % ethanol (to adjust RNA binding conditions)
- Reducing agent (ß-mercaptoethanol, **or** DTT (dithiothreithol), **or** TCEP (BisTris (Bis-(2-hydroxyethyl)-imino-tris(hydroxymethyl)-methane)) as supplement for Lysis Buffer RA1

Consumables

- 1.5 mL microcentrifuge tubes (NucleoSpin® RNA) or 15 mL tubes (NucleoSpin® RNA Midi)
- Sterile RNase-free tips

Equipment

- Manual pipettors
- NucleoSpin® RNA: centrifuge for microcentrifuge tubes
- NucleoSpin® RNA Midi: centrifuge for 15 mL tubes with a swing-out rotor and appropriate buckets capable of reaching 4,000–4,500 x *g*
- Equipment for sample disruption and homogenization (see section 2.3)
- Personal protection equipment (e.g., lab coat, gloves, goggles)

1.3 About this user manual

It is strongly recommended reading the detailed protocol sections of this user manual if the **NucleoSpin® RNA** or **NucleoSpin® RNA Midi** kit is used for the first time. Experienced users, however, may refer to the Protocol-at-a-glance instead. The Protocol-at-a-glance is designed to be used only as a supplemental tool for quick referencing while performing the purification procedure.

All technical literature is available on the Internet at ***www.mn-net.com***.

Please contact Technical Service regarding information about changes of the current user manual compared to previous revisions.

2 Product description

2.1 The basic principle

One of the most important aspects in the isolation of RNA is to prevent degradation during the isolation procedure. With the **NucleoSpin® RNA** methods, cells are lysed by incubation in a solution containing large amounts of chaotropic ions. This lysis buffer immediately inactivates RNases – which are present in virtually all biological materials – and creates appropriate binding conditions which favor adsorption of RNA to the silica membrane. Contaminating DNA, which is also bound to the silica membrane, is removed by an rDNase solution which is directly applied onto the silica membrane during the preparation (RNase-free rDNase is supplied with the kit). Simple washing steps with two different buffers remove salts, metabolites and macromolecular cellular components. Pure RNA is finally eluted under low ionic strength conditions with RNase-free H_2O (supplied).

The RNA preparation using **NucleoSpin® RNA** kits can be performed at room temperature. The eluate, however, should be treated with care because RNA is very sensitive to trace contaminations of RNases, often found on general lab ware, fingerprints and dust. To ensure RNA stability keep RNA frozen at -20 °C for short-term or -70 °C for long-term storage.

Simultaneous isolation of RNA, Protein, and DNA (NucleoSpin® RNA / DNA Buffer Set*, NucleoSpin® TriPrep*)

The NucleoSpin® RNA / DNA Buffer Set (see ordering information) is a support set for RNA and DNA isolation in conjunction with NucleoSpin® RNA, NucleoSpin® RNA XS, NucleoSpin® RNA Plant, or NucleoSpin® RNA / Protein.

This patented technology enables successive elution of DNA and RNA from one NucleoSpin® Column with low salt buffer and water respectively. DNA and RNA are immediately ready for downstream applications.

The combination of the NucleoSpin® RNA / DNA Buffer Set with NucleoSpin® RNA / Protein allows parallel isolation of RNA, DNA, and protein from one undivided sample.

The NucleoSpin® TriPrep kit features the purification of RNA, DNA, and protein from single undivided samples.

2.2 Kit specifications

- **NucleoSpin® RNA** kits are recommended for the isolation of RNA from cultured cells and tissue. Support protocols for the isolation of RNA from cell-free biological fluids, bacteria, and yeasts using the **NucleoSpin® RNA** kit are included. The **NucleoSpin® RNA** kits allow purification of pure RNA with an A_{260}/A_{280} ratio generally exceeding 1.9 (measured in TE buffer, pH 7.5).
- Even biological samples which are sometimes difficult to process will yield high quality RNA. Such samples are, for example, mouse tissue (liver, brain), different tumor cell lines, *Streptococci*, and *Actinobacillus pleuropneumoniae*.
- The isolated RNA is ready to use for applications like reverse transcriptase-PCR (RT-PCR), primer extension, or RNase protection assays.
- RNA isolated with NucleoSpin® RNA kits is of high integrity. RIN (RNA Integrity Number) of RNA isolated from fresh high quality sample material (e.g., eukaryotic cells or fresh mouse liver) generally exceeds 9.0. However, RNA integrity strongly depends on the sample quality. RNA integrity was examined using the Agilent 2100 Bioanalyzer in conjunction with the RNA 6000 Nano or Pico assay.
- The amount of DNA contamination is significantly reduced during on-column digestion with rDNase. Anyhow, in very sensitive applications it might be possible to detect traces of DNA. The **NucleoSpin® RNA** on-column DNA removal is tested with the following procedure: One million HeLa cells are subjected to RNA isolation according to the protocol. RNA eluate is used as template for PCR detection of a 1 kb fragment in a 30 cycle reaction. Generally no PCR fragment is obtained if the DNase is applied while a strong PCR fragment may be obtained if the DNase digestion is omitted. The probability of DNA detection with PCR increases with:
  1. the number of DNA copies per preparation: single copy target < plastidial/mitochondrial target < plasmid transfected into cells.
  2. decreasing PCR amplicon size.

Table 1: Kit specifications at a glance

Parameter	NucleoSpin® RNA	NucleoSpin® RNA Midi
Technology	Silica-membrane technology	Silica-membrane technology
Format	Mini spin column	Midi spin column
Sample material	< 5 x 10^6 cultured cells, < 10^9 bacterial cells, < 10^8 yeast cells, < 30 mg tissue	< 5 x 10^7 cultured cells, < 10^{10} bacterial cells, < 3 x 10^8 yeast cells, < 200 mg tissue
Fragment size	> 200 nt	> 200 nt
Typical yield	14 µg from 10^6 HeLa cells, 70 µg from 10^9 bacterial cells	180 µg from 10^7 HeLa cells, 620 µg from 4 x 10^7 HeLa cells
A_{260}/A_{280}	1.9–2.1	1.9–2.1
Typical RIN (RNA integrity number)	> 9	> 9
Elution volume	40–120 µL	500 µL
Preparation time	30 min/6 preps	80 min/4 preps
Binding capacity	200 µg	700 µg

NucleoSpin® RNA

- The standard protocol (section 5.1) allows the purification of up to 70 µg RNA per **NucleoSpin® RNA Column** from up to 5 x 10^6 cultured cells or 30 mg of tissue (also see Table 1). The isolated RNA can be used as template in a RT-PCR-reaction. Generally, 1–10 % of the eluate of RNA prepared from 1 x 10^6 cells or 10 mg of tissue is sufficient as template for RT-PCR. If possible, intronspanning primers should be used for RT-PCR.
- The RNA prepared from such high amounts is generally free of residual DNA, although minute traces of DNA may remain in the preparation, if large amounts of material rich in nucleic acids are used. However, if the isolated RNA will be used as template in a RT-PCR-reaction, we recommend to use lower quantities of sample (e.g., 1 x 10^6 cultured cells or 10 mg of tissue resulting in about 20 µg of RNA).
- The kit can be used for preparing RNA from different amounts of sample material. For optimal results the volume of Lysis Buffer RA1 (protocol step 1) and of ethanol (protocol step 3) should be adapted according to Table 2.

Table 2: Lysis adaptation

Sample	Amount	Volume of Lysis Buffer RA1 (protocol step 2)	Volume of Ethanol (protocol step 4)
Cultured animal or human cells (e.g., HeLa cells)	< 5 x 10^6	350 µL	350 µL
Human or animal tissue	< 20 mg 20 mg–30 mg*	350 µL 600 µL	350 µL 600 µL
Tissue stored in RNA*later*®	< 20 mg 20 mg–30 mg*	350 µL 600 µL	350 µL 600 µL
Samples known to be hard to lyse	< 5 x 10^7*	600 µL	600 µL

An additional loading step is required if 600 µL Buffer RA1 and ethanol is used (load the sample onto the column in two successive centrifugation steps).

Depending on sample type, the average yield is around 5–70 µg RNA (see Table 3). The A_{260}/A_{280} ratio generally exceeds 1.9, indicating purity of the RNA.

Table 3: Overview on average yields of RNA isolation using NucleoSpin® RNA

Sample	Average yield
8 x 10^4 HeLa cells	1.5 µg
4 x 10^5 HeLa cells	4 µg
1 x 10^6 HeLa cells	14 µg
2 x 10^6 HeLa cells	21 µg
2.5 x 10^6 HeLa cells	25 µg
5 x 10^6 HeLa cells	50 µg

* The volume of Lysis Buffer RA1 included in the kit is not sufficient to perform all preparations with 600 µL. If required, additional Lysis Buffer RA1 can be ordered separately (see ordering information, section 8.2).

NucleoSpin® RNA Midi

- The kit can be used for preparing RNA from different amounts of sample material. For optimal results the volume of Lysis Buffer RA1 (protocol step 1) and of ethanol (protocol step 3) should be adapted according to Table 4:

Table 4: Lysis adaptation

Sample	Amount	Volume of Lysis Buffer RA1 (protocol step 1)	Volume of Ethanol (protocol step 4)
Cultured animal cells (e.g., HeLa cells)	5 x 10^6–2 x 10^7	1.8 mL	1.8 mL
	2 x 10^7–5 x 10^7	3.6 mL	3.6 mL
Animal tissue	30–100 mg	1.8 mL	1.8 mL
	100–200 mg	3.6 mL	3.6 mL
Bacteria	1 x 10^9–5 x 10^9	1.8 mL	1.8 mL
	2 x 10^9–1 x 10^{10}	3.6 mL	3.6 mL
Yeast	< 3 x 10^8	3.6 mL	3.6 mL

An additional loading step is required if 3.6 mL Buffer RA1 and ethanol is used. If you isolate RNA from a certain kind of tissue the first time with the NucleoSpin® RNA Midi kit, we recommend starting with no more than 100 mg of tissue. Depending on the nature of the tissue, up to 200 mg can be processed. Do not use more than 200 mg of tissue to avoid clogging of the column.

Depending on sample type, the average yield is around 70–400 µg RNA (see Table 5). The A_{260}/A_{280} ratio indicating purity of the RNA generally exceeds 1.9.

Table 5:
Overview on average yields of RNA isolation using NucleoSpin® RNA Midi

Sample	Average yield
1 x 10^6 HeLa cells	20 µg
1 x 10^7 HeLa cells	160 µg
2 x 10^7 HeLa cells	330 µg
4 x 10^7 HeLa cells	620 µg
200 mg pig liver	450 µg
200 mg mouse liver	320 µg

2.3 Handling, preparation, and storage of starting materials

RNA is not protected against digestion until the sample material is flash frozen or disrupted in the presence of RNase inhibiting or denaturing agents. Therefore it is important that samples are flash frozen in liquid N_2 immediately and stored at -70 °C or processed as soon as possible. Frozen samples are stable up to 6 months. Samples can be stored in Lysis Buffer RA1 after disruption at -70 °C for up to one year, at 4 °C for up to 24 hours or up to several hours at room temperature. Frozen samples in Buffer RA1 should be thawed slowly before starting with the isolation of RNA.

Wear gloves at all times during the preparation. Change gloves frequently.

Cultured animal cells are collected by centrifugation and directly lysed by adding Buffer RA1 according to step 2 of the standard protocol (see sections 5.1, 6.1).

Cell lysis of adherent growing cells in a culture dish:

Completely aspirate cell-culture medium, and continue immediately with the addition of Lysis Buffer RA1 to the cell-culture dish. Avoid incomplete removal of the cell-culture medium in order to allow full lysis activity of the lysis buffer.

To trypsinize adherent growing cells:

Aspirate cell-culture medium, and add an equal amount of PBS in order to wash the cells. Aspirate PBS. Add 0.1–0.3 % trypsin in PBS and incubate for an appropriate time to detach the cells from the dish surface. After cell detachment, add medium, transfer cells to an appropriate tube (not supplied), and pellet by centrifugation for 5 min at 300 x *g*. Remove supernatant and continue with the addition of lysis buffer to the cell pellet.

Animal tissues are often solid and must therefore be broken up mechanically as well as lysed. Depending on the disruption method, the viscosity of the lysed sample has to be reduced further for optimal results. It is essential for efficient RNA preparation that all the RNA contained in the sample is released from the cells by disruption and that the viscosity of the sample is reduced by homogenization.

The most commonly used technique for disruption of animal tissues is grinding with a pestle and mortar. Grind the sample to a fine powder in the presence of liquid N_2. Take care that the sample does not thaw during or after grinding or weighing and add the frozen powder to an appropriate aliquot of Buffer RA1 containing reducing agent, (e.g., ß-mercaptoethanol, DTT, or TCEP) and mix immediately. The broken-up tissue must then be homogenized with a **NucleoSpin® Filter / Filter Midi** (included in the kit) or by passing ≥ 5 times through a 0.9 mm syringe needle.

Thawing of undisrupted animal tissue should be exclusively done in the presence of Buffer RA1 during simultaneous mechanical disruption, for example, with a **rotor-stator homogenizer**. This ensures that the RNA is not degraded by RNases before the preparation has started. The spinning rotor disrupts and simultaneously homogenizes the sample by mechanical shearing of DNA within seconds up to minutes (homogenization time depends on sample). Take care to keep the rotor tip submerged in order to avoid excess foaming. Select a suitably sized homogenizer (5–7 mm diameter rotors can be used for homogenization in microcentrifuge tubes).

Bacteria and yeasts have to be incubated in lysozyme or lyticase / zymolase solutions, respectively (see support protocols in section 5.2, 5.3). By this treatment, the robust cell walls of these organisms are digested or at least weakened, which is essential for effective cell lysis by Buffer RA1. For microorganisms with extremely resistant cell walls – like some Gram-positive bacterial strains – it may be necessary to optimize the conditions of the treatment with lytic enzymes or the cultivation conditions. After lysis, homogenization is achieved by the use of a **NucleoSpin® Filter** or the syringe-needle method.

2.4 Elution procedures

It is possible to adapt elution method and volume of water used for the subsequent application of interest. In addition to the standard method described in the individual protocols (recovery rate about 70–90 %) there are several modifications possible.

- **High yield:** Perform two elution steps with the volume indicated in the individual protocol. About 90–100 % of bound nucleic acid will be eluted.
- **High yield and high concentration:** Elute with the standard elution volume and apply the eluate once more onto the column for reelution.

Eluted RNA should immediately be put and always kept on ice for optimal stability because almost omnipresent RNases (general lab ware, fingerprints, dust) will degrade RNA. For short-term storage freeze at -20 °C, for long-term storage freeze at -70 °C.

3 Storage conditions and preparation of working solutions

Attention:

Buffers RA1, RAW2, and MDB contain chaotropic salt. Wear gloves and goggles!

CAUTION: Buffers RA1, RAW2 and MDB contain chaotropic salt which can form highly reactive compounds when combined with bleach (sodium hypochlorite). DO NOT add bleach or acidic solutions directly to the sample-preparation waste.

- Store lyophilized **rDNase (RNase-free)** at 4 °C on arrival (stable up to 1 year).
- All other kit components should be stored at room temperature (18–25 °C) and are stable for at least one year. Storage at lower temperatures may cause precipitation of salts.
- Check that 70 % ethanol is available as additional solution to adjust RNA binding conditions in the lysate.
- Check that reducing agent (ß-ME, DTT, or TCEP) is available.

Before starting any **NucleoSpin® RNA** protocol, prepare the following:

- **rDNase (RNase-free):** Add indicated volume of RNase-free H_2O (see table below) to the rDNase vial and incubate for 1 min at room temperature. Gently swirl the vials to completely dissolve the rDNase. Be careful not to mix rDNase vigorously as rDNase is sensitive to mechanical agitation. Dispense into aliquots and store at -20 °C. The frozen working solution is stable for at least 6 months. Do not freeze / thaw the aliquots more than three times. (Be careful when opening the vial as some particles of the lyophilisate may be attached to the lid.)

In some cases the vial of rDNase may appear empty. This is due to lyophilized enzyme sticking to the septum. To avoid loss of rDNase, make sure to collect rDNase on the bottom of the vial before removing the plug. Alternatively, inject RNase-free water into the vial using a needle and syringe, invert the vial to dissolve the rDNase, and remove the dissolved rDNase using syringe and needle.

- **Wash Buffer RA3:** Add the indicated volume of 96–100 % ethanol (see table on next page) to Buffer RA3 Concentrate. Mark the label of the bottle to indicate that ethanol was added. Wash Buffer RA3 can be stored at room temperature (18–25 °C) for at least one year.

	NucleoSpin® RNA		
REF	**10 preps** **740955.10**	**50 preps** **740955.50**	**250 preps** **740955.250**
Wash Buffer RA3 (Concentrate)	6 mL Add 24 mL ethanol	12 mL Add 48 mL ethanol	3 x 25 mL Add 100 mL ethanol to each vial
rDNase, RNase-free (lyophilized)	1 vial (size D) Add 120 µL RNase-free H_2O	1 vial (size F) Add 550 µL RNase-free H_2O	5 vials (size F) Add 550 µL RNase-free H_2O to each vial

	NucleoSpin® RNA Midi
REF	**20 preps** **740962.20**
Wash Buffer RA3 (Concentrate)	25 mL Add 100 mL ethanol
rDNase, RNase-free (lyophilized)	1 vial (size D) Add 540 µL RNase-free H_2O

4 Safety instructions

The following components of the NucleoSpin® RNA and NucleoSpin® RNA Midi kits contain hazardous contents.

Wear gloves and goggles and follow the safety instructions given in this section.

GHS classification

Only harmful features do not need to be labeled with H and P phrases up to 125 mL or 125 g.
Mindergefährliche Eigenschaften müssen bis 125 mL oder 125 g nicht mit H- und P-Sätzen gekennzeichnet werden.

Component *Inhalt*	Hazard contents *Gefahrstoff*	GHS symbol *GHS-Symbol*	Hazard phrases *H-Sätze*	Precaution phrases *P-Sätze*
rDNase, RNase-free	rDNase, lyophilized *rDNase, lyophilisiert* CAS 9003-98-9	 DANGER *GEFAHR*	317, 334	261, 280, 302+352, 304+340, 333+313, 342+311, 363
RA1	guanidinium thiocyanate 30–60 % *Guanidinthiocyanat 30–60 %* CAS 593-84-0	 WARNING *ACHTUNG*	302, 412, EUH031	260, 273, 301+312, 330
RAW2	guanidinium thiocyanate 24–36 % and ethanol 20–35 % *Guanidinhydrochlorid 24–36 % und Ethanol 20–35 %* CAS 50-01-1, 64-17-5	 WARNING *ACHTUNG*	226, 302	210, 233, 301+312, 330, 370+378, 403+235
MDB	ethanol 5–15 % *Ethanol 5–15 %* CAS 64-17-5	 WARNING *ACHTUNG*	226	210, 233, 370+378, 403+235

Hazard phrases

H 226 Flammable liquid and vapor.
Flüssigkeit und Dampf entzündbar.

H 302 Harmful if swallowed.
Gesundheitsschädlich bei Verschlucken.

H 317 May cause an allergic skin reaction.
Kann allergische Hautreaktionen verursachen.

H 334 May cause allergy or asthma symptoms or breathing difficulties if inhaled.
Kann bei Einatmen Allergie, asthmaartige Symptome oder Atembeschwerden verursachen.

H 412	Harmful to aquatic life with long lasting effects. *Gesundheitsschädlich bei Verschlucken.*
EUH031	Contact with acids liberates toxic gas. *Schädlich für Wasserorganismen, mit langfristiger Wirkung. Entwickelt bei Berührung mit Säure giftige Gase.*

Precaution phrases

P 210	Keep away from heat / sparks / open flames / hot surfaces. No smoking. *Von Hitze / Funken / offener Flamme / heißen Oberflächen fernhalten. Nicht rauchen.*
P 233	Keep container tightly closed. *Behälter dicht verschlossen halten.*
P260	Do not breathe vapors. *Dampf nicht einatmen.*
P261	Avoid breathing dust. *Einatmen von Staub vermeiden.*
P272	Contaminated work clothing should not be allowed out of the workplace. *Kontaminierte Arbeitskleidung nicht außerhalb des Arbeitsplatzes tragen.*
P273	Avoid release to the environment. *Freisetzung in die Umwelt vermeiden*
P280	Wear protective gloves/eye protection. *Schutzhandschuhe/Augenschutz tragen.*
P301+312	IF SWALLOWED: Call a POISON CENTER/doctor/.../if you feel unwell. *BEI VERSCHLUCKEN: Bei Unwohlsein GIFTINFORMATIONSZENTRUM/Arzt/… anrufen.*
P302+352	IF ON SKIN: Wash with plenty of water/… *BEI BERÜHRUNG MIT DER HAUT: Mit viel Wasser/… waschen.*
P304+340	IF INHALED: Remove person to fresh air and keep comfortable for breathing. *BEI EINATMEN: Die Person an die frische Luft bringen und für ungehinderte Atmung sorgen.*
P330	Rinse mouth. *Mund ausspülen.*
P333+313	If skin irritation or rash occurs: Get medical advice/attention. *Bei Hautreizung oder -ausschlag: Ärztlichen Rat einholen/ärztliche Hilfe hinzuziehen.*
P342+311	If experiencing respiratory symptoms: Call a POISON CENTER/doctor/… *Bei Symptomen der Atemwege: GIFTINFORMATIONSZENTRUM/Arzt/… anrufen.*
P363	Wash contaminated clothing before reuse. *Kontaminierte Kleidung vor erneutem Tragen waschen.*
P370+378	In case of fire: Use all extinguisher media to extinguish. *Bei Brand: Alle Löschmittel zum Löschen verwenden.*
P403+235	Store in a well-ventilated place. Keep cool. *Kühl an einem gut belüfteten Ort aufbewahren.*

For further information please see Material Safety Data Sheets (***www.mn-net.com***).
*Weiterführende Informationen finden Sie in den Sicherheitsdatenblättern (**www.mn-net.com**).*

5 NucleoSpin® RNA protocols

5.1 RNA purification from cultured cells and tissue

Before starting the preparation:

- Check if Wash Buffer RA3 and rDNase were prepared according to section 3.

1 Homogenize sample

Disrupt sample

Disrupt up to **30 mg** of **tissue** (for sample amounts see section 2.2; for homogenization methods see section 2.3).

Up to **5 x 10**^{6} eukaryotic cultured cells can be collected by centrifugation and lysed by addition of Buffer RA1 directly.

2 Lyse cells

+ 350 µL RA1

+ 3.5 µL ß-ME

Add **350 µL Buffer RA1** and **3.5 µL ß-mercaptoethanol** (ß-ME) to the cell pellet or to ground tissue and vortex vigorously.

For appropriate sample and lysis buffer amounts see section 2.2.

Note: As alternative to ß-ME the reducing agent DTT or TCEP may be used. Use a final concentration of 10–20 mM DTT or TCEP within the Lysis Buffer RA1.

3 Filtrate lysate

11,000 x g, 1 min

Reduce viscosity and clear the lysate by filtration through **NucleoSpin® Filter (violet ring)**: Place NucleoSpin® Filter in a Collection Tube (2 mL), apply the mixture, and centrifuge for **1 min** at **11,000 x *g***.

The lysate may be passed alternatively ≥ 5 times through a 0.9 mm needle (20 gauge) fitted to a syringe.

In case of visible pellet formation (depending on sample amount and nature) transfer supernatant without any formed pellet to a new 1.5 mL microcentrifuge tube (not supplied).

Important: To process higher amounts of cells (> 1 x 10^6) or tissue (> 10 mg), the lysate should first be homogenized using the 0.9 mm needle (20 gauge), followed by filtration through NucleoSpin® Filters.

4 Adjust RNA binding conditions

Discard the NucleoSpin® Filter and add **350 µL ethanol (70 %)** to the homogenized lysate and mix by pipetting up and down (5 times).

+ 350 µL 70 % ethanol

Mix

Alternatively, transfer flow-through into a new 1.5 mL microcentrifuge tube (not provided), add **350 µL ethanol (70 %)**, and mix by vortexing (2 x 5 s).

After addition of ethanol a stringy precipitate may become visible which will not affect the RNA isolation. Be sure to disaggregate any precipitate by mixing and load all of the precipitate on the column as described in step 5. Do not centrifuge the ethanolic lysate before loading it onto the column in order to avoid pelleting the precipitate.

5 Bind RNA

For each preparation take one **NucleoSpin® RNA Column (light blue ring)** placed in a Collection Tube. Pipette lysate up and down 2–3 times and **load the lysate** to the column. Centrifuge for **30 s** at **11,000 x *g***. Place the column in a new Collection Tube (2 mL).

Load lysate

11,000 x *g*, 30 s

Maximal loading capacity of NucleoSpin® RNA Columns is 750 µL. Repeat the procedure if larger volumes are to be processed.

6 Desalt silica membrane

Add **350 µL MDB** (Membrane Desalting Buffer) and centrifuge at **11,000 x *g*** for **1 min** to dry the membrane.

+ 350 µL MDB

11,000 x *g*, 1 min

Salt removal will make the following rDNase digest much more effective. If the column outlet has come into contact with the flow-through for any reason, discard the flow-through and centrifuge again for 30 s at 11,000 x *g*.

7 Digest DNA

Prepare DNase reaction mixture in a sterile 1.5 mL microcentrifuge tube (not provided): For each isolation, add **10 µL reconstituted rDNase** (also see section 3) to **90 µL Reaction Buffer for rDNase**. Mix by flicking the tube.

+ 95 µL rDNase reaction mixture

RT, 15 min

Apply **95 µL DNase reaction mixture** directly onto the center of the silica membrane of the column. Incubate at **room temperature** for 1**5 min**.

8 Wash and dry silica membrane

1st wash

Add **200 µL Buffer RAW2** to the NucleoSpin® RNA Column. Centrifuge for **30 s** at **11,000 x *g***. Place the column into a new Collection Tube (2 mL).

Buffer RAW2 will inactivate the rDNase.

+ 200 µL RAW2

11,000 x *g*, 30 s

2nd wash

Add **600 µL Buffer RA3** to the NucleoSpin® RNA Column. Centrifuge for **30 s** at **11,000 x *g***. Discard flow-through and place the column back into the Collection Tube.

Note: Make sure that residual buffer from the previous steps is washed away with Buffer RA3, especially if the lysate has been in contact with the inner rim of the column during loading of the lysate onto the column. For efficient washing of the inner rim flush it with Buffer RA3.

+ 600 µL RA3

11,000 x *g*, 30 s

3rd wash

Add **250 µL Buffer RA3** to the NucleoSpin® RNA Column. Centrifuge for **2 min** at **11,000 x *g*** to dry the membrane completely. Place the column into a nuclease-free Collection Tube (1.5 mL, supplied).

If for any reason, the liquid level in the Collection Tube has reached the NucleoSpin® RNA Column after centrifugation, discard flow-through, and centrifuge again.

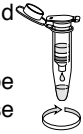
+ 250 µL RA3

11,000 x *g*, 2 min

9 Elute RNA

Elute the RNA in **60 µL RNase-free H_2O**, (supplied) and centrifuge at **11,000 x *g*** for 1 min.

If higher RNA concentrations are desired, elution can be done with 40 µL. Overall yield, however, will decrease when using smaller volumes.



+ 60 µL RNase-free H2O

11,000 x *g*, 1 min

For further alternative elution procedures see section 2.4.

5.2 RNA preparation from up to 10^9 bacterial cells

Additional reagent to be supplied by user:

- Lysozyme

Before starting the preparation:

- Check if Wash Buffer RA3 and rDNase were prepared according to section 3.

1 Homogenize sample

Resuspend the **bacterial cell pellet** (Gram-negative strains) in **100 µL TE buffer** (10 mM Tris-HCl, 1 mM EDTA; pH 8) containing **1 mg/mL lysozyme** by vigorous vortexing. Incubate at **37 °C** for **10 min**.

For preparation of RNA from Gram-positive bacteria, resuspend cells in 100 µL TE containing 2 mg/mL lysozyme. It may be necessary to optimize incubation time and lysozyme concentration, depending on the bacterial strain.

Note: Due to the much higher concentration of genome equivalents in a nucleic acid preparation of bacteria compared with eukaryotic material, it may be necessary to use a lower quantity of cells for the preparation.

2 Lyse cells

Add **350 µL Buffer RA1** and **3.5 µL ß-mercaptoethanol** to the suspension and vortex vigorously.

For appropriate sample and lysis buffer amounts see section 2.2.

Note: As alternative to ß-ME the reducing agent DTT or TCEP may be used. Use a final concentration of 10–20 mM DTT or TCEP within the Lysis Buffer RA1.

3 Filtrate lysate

Reduce viscosity and turbidity of the solution by filtration through **NucleoSpin® Filters (violet rings)**. Place NucleoSpin® Filters in Collection Tubes (2 mL), apply mixture, and centrifuge for **1 min** at **11,000 x *g***.

In case of visible pellet formation (depending on sample amount and nature) transfer supernatant without any formed pellet to a new 1.5 mL microcentrifuge tube (not supplied).

Alternatively, the lysate may be passed ≥ 5 times through a 0.9 mm needle (20 gauge) fitted to a syringe.

Adjust RNA binding conditions

Add **350 µL of ethanol (70 %)** to the lysate and mix by vortexing.

Proceed with step 5 of the NucleoSpin® RNA standard protocol (section 5.1)

5.3 RNA preparation from up to 5 x 10^7 yeast cells

Additional reagents and components to be supplied by user:

- Reducing agent (ß-mercaptoethanol, or DTT (dithiothreithol), **or** TCEP (BisTris (Bis-(2-hydroxyethyl)-imino-tris(hydroxymethyl)-methane))
- Sorbitol and lyticase (or zymolase) for homogenization by enzymatic digestion **or** a swing-mill and glass beads for homogenization by mechanical disruption

Before starting the preparation:

- Check if Wash Buffer RA3 and rDNase were prepared according to section 3.

1 Homogenize sample

Two alternative protocols are given for homogenization of yeast cells. Users may choose between an enzymatic digestion (A) **or** mechanical homogenization (B), depending on laboratory equipment and personal preference. Homogenization by enzymatic digest is only recommended for fresh harvested cells, homogenization by mechanical disruption may also be performed with yeast cell pellets, stored at -70 °C for several months.

Note: Due to the much higher concentration of genome equivalents in a nucleic acid preparation of yeasts compared with cultured cells or tissue material, it may be necessary to use a lower quantity of cells for the preparation.

A) Homogenization by enzymatic digest

Harvest 2–5 mL of **YPD culture (5,000 x *g*; 10 min)**. Resuspend pellet in an appropriate amount of fresh prepared sorbitol / lyticase buffer (50–100 U lyticase or zymolase in 1 M sorbitol / 100 mM EDTA) and incubate at **30 °C** for **30 min**. Pellet the resulting spheroplasts by centrifugation **(1,000 x *g*; 10 min)**.

Carefully discard supernatant.

It may be necessary to optimize incubation time and lyticase / zymolase concentration, depending on the yeast strain.

Continue with step 2.

OR

B) Homogenization by mechanical disruption

Harvest 2–5 mL of **YPD culture (5,000 x *g*; 10 min)** and wash with ice-cold water. Resuspend the cell pellet in a mixture of 350 µL Buffer RA1 and 3.5 µL ß-mercaptoethanol.

Add glass beads (e.g., 300 mg glass beads, 425–600 µm, Sigma-Aldrich # 68772).

Shake samples in a swing-mill at 30 Hz for **15 min**.

Continue with step 3.

Note: As alternative to ß-ME the reducing agent DTT or TCEP may be used. Use a final concentration of 10–20 mM DTT or TCEP within the Lysis Buffer RA1.

2 Lyse cells

Add **350 µL Buffer RA1** and **3.5 µL ß-mercaptoethanol** and vortex vigorously to lyse spheroplasts.

For appropriate sample and lysis buffer amounts see section 2.2.

Note: As alternative to ß-ME the reducing agent DTT or TCEP may be used. Use a final concentration of 10–20 mM DTT or TCEP within the Lysis Buffer RA1.

3 Filtrate lysate

Reduce viscosity and turbidity of the solution by filtration through **NucleoSpin® Filters (violet rings)**. Place NucleoSpin® Filters in Collection Tubes (2 mL), apply mixture, and centrifuge for **1 min** at **11,000 x *g***.

In case of visible pellet formation (depending on sample amount and nature) transfer supernatant without any formed pellet to a new 1.5 mL microcentrifuge tube (not supplied).

Alternatively, the lysate may be passed ≥ 5 times through a 0.9 mm needle (20 gauge) fitted to a syringe.

4 Adjust RNA binding conditions

Add **350 µL of ethanol (70 %)** to the lysate and mix by vortexing.

Proceed with step 5 of the NucleoSpin® RNA standard protocol (section 5.1).

5.4 RNA preparation from paraffin embedded tissue*

Additional reagent to be supplied by user:

- Xylene

Before starting the preparation:

- Check if Wash Buffer RA3 and rDNase were prepared according to section 3.

A	Put **10 mg** of finely minced tissue into a 1.5 mL microcentrifuge tube (not provided). Add **300 µL xylene** and incubate 5 min with constant mixing at room temperature.
B	Centrifuge at **maximum speed** (13,000 rpm) for **3 min** to pellet the tissue. Discard the xylene.
C	Repeat the steps A and B twice, for a total of three xylene washes.
D	Add **300 µL of 96 % ethanol** to the tube and incubate **5 min** with constant mixing at **room temperature**.
E	Centrifuge at **maximum speed** (13,000 rpm) for **3 min** to pellet the tissue. Discard the ethanol.
F	Repeat steps D and E, for a total of two ethanol washes.
	Continue with step 1 of the NucleoSpin® RNA standard protocol (section 5.1). *<u>Note:</u> For high performance isolation of RNA from formalin-fixed, paraffin-embedded tissue the NucleoSpin® totalRNA FFPE (REF 740982, see ordering information, section 8.2) or NucleoSpin® totalRNA FFPE XS (REF 740969, see ordering information, section 8.2) is recommended.*

* Please also refer to: Annunziata Gloghini, Barbara Canal, Ulf Klein, Luigino Dal Maso, Tiziana Perin, Riccardo Dalla-Favera, and Antonino Carbone **RT-PCR Analysis of RNA Extracted from Bouin-Fixed and Paraffin-Embedded Lymphoid Tissues** J Mol Diagn 2004 6: 290–296 as one example for customer modification of the support protocol mentioned above.

5.5 Clean-up of RNA from reaction mixtures

Before starting the preparation:

- Check that Wash Buffer RA3 was prepared according to section 3.

1 Prepare sample

Fill up RNA samples smaller than 100 µL with RNase-free H_2O to 100 µL.

If different samples with varying volumes between 100 and 200 µL are purified, RNA samples should be filled up with RNase-free H_2O to a uniform volume (e.g., 200 µL).

2 Prepare lysis-binding buffer premix

Prepare a Buffer RA1 - ethanol premix with ratio 1 : 1.

For **each 100 µL RNA sample,** mix **300 µL Buffer RA1** and **300 µL ethanol (96–100 %)**.

If multiple samples are processed, the preparation of a master-premix is recommended (e.g., 2 mL RA1 + 2 mL 98 % ethanol for approximately 6 NucleoSpin® RNA preparations).

3 Filtrate lysate

Not necessary!

4 Adjust RNA binding conditions

To **100 µL of RNA sample, add 600 µL (6 volumes) of Buffer RA1 - ethanol premix**. Mix sample with premix by vortexing.

If 200 µL of RNA samples are processed, add 1200 µL of RA1-ethanol premix.

Maximal loading capacity of NucleoSpin® RNA Columns is 750 µL. Repeat the procedure if larger volumes are to be processed.

After addition of ethanol, a stringy precipitate may become visible which will not affect the RNA isolation. Be sure to mix thoroughly and apply sample as homogenieous solution onto the column. For binding capacity of the columns, see Table 1.

Proceed with step 5, 8, and 9 of the NucleoSpin® RNA standard protocol (section 5.1). Steps 6 and 7 of the respective protocols may be omitted in this case.

As alternative products for RNA clean-up, NucleoSpin® RNA Clean-up and NucleoSpin® RNA Clean-up XS are recommended (see ordering information, section 8.2*).*

6 NucleoSpin® RNA Midi protocols

6.1 RNA purification from cultured cells and tissue

Before starting the preparation:

- Check that Wash Buffer RA3 and rDNase were prepared according to section 3.
- For centrifugation, a centrifuge with a **swing-out rotor** and appropriate buckets capable of reaching 4,000–4,500 x *g* is required.

1 Homogenize sample

Disrupt sample

Disrupt up to 100 mg of tissue (for sample amounts see section 2.2; for homogenization methods see section 2.3).

Up to **4 x 10^7** eukaryotic **cultured cells** are collected by centrifugation and lysed by addition of Buffer RA1 directly.

To choose an appropriate amount of starting material see section 2.2***.***

2 Lyse cells

+ 1.8 mLRA1

+ 18 µL ß-ME

Add **1.8 mL Buffer RA1** and **18 µL ß-mercaptoethanol** (ß-ME) to the disrupted material in a 15 mL centrifuge tube (not supplied) and vortex vigorously (use 3.6 mL Buffer RA1 and 36 µL ß-mercaptoethanol for large sample amounts; see section 2.2.)

Note: As alternative to ß-ME the reducing agent DTT or TCEP may be used. Use a final concentration of 10–20 mM DTT or TCEP within the Lysis Buffer RA1.

3 Filtrate lysate

4,500 x *g*, 10 min

Apply the lysate to a **NucleoSpin® Filter Midi** placed in a Collection Tube and centrifuge sample for **10 min** at **4,500 x *g***. This step will homogenize the sample by removal of residual insoluble material and simultaneous reduction of lysate viscosity.

In case of visible pellet formation (depending on sample amount and nature) transfer supernatant without any formed pellet to a new 15 mL centrifuge tube (not supplied).

If working with small amounts of cultured cells (e.g., < 1 x 10^7 HeLa cells) step 3 may be substituted by vigorous mixing of the sample.

4 Adjust RNA binding conditions

Discard the NueoSpin® Filter Midi and add **1.8 mL ethanol (70 %)** to the lysate in the Collection Tube and mix by vortexing 2 x 5 s (use 3.6 mL of 70 % ethanol if working with large sample amounts, see step 2 and section 2.2).

After addition of ethanol a stringy precipitate may become visible which will not affect the further procedure. Resuspend precipitates thoroughly before loading onto the NucleoSpin® RNA Midi Column.

+ 1.8 mL 70 % ethanol

Mix

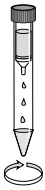
5 Bind RNA

Load the lysate-ethanol mixture (maximal 3.8 mL) onto a **NucleoSpin® RNA Midi Column**. Centrifuge for **3 min** at **4,500 x *g***.

If working with large sample amounts, apply the rest of the lysate-ethanol mixture (max. 3.8 mL) onto the column and centrifuge again.

If the lysate has not passed through the column, centrifuge again at 4,500 x g for 10 min.

In case of column-overloading incomplete flow-through of the sample might be observed, for example, the membrane is still wet or some lysate has not passed through. Remove the lysate, which has not passed through the column, and continue with the next protocol step. Use less starting material and carefully remove insoluble material in step 3 next time.



Load max. 3.8 mL lysate

4,500 x g, 3 min

6 Desalt silica membrane

Add **2.2 mL MDB** (Membrane Desalting Buffer) to the NucleoSpin® RNA Midi Column. Centrifuge for 3 min at **4,500 x *g***. Discard flow-through.

If the silica membrane is not completely dry after centrifugation, centrifuge again at 4,500 x *g* for 10 min. This step will create optimal reaction conditions for the rDNase.

+ 2.2 mL MDB

4,500 x g, 3 min

7 Digest DNA

Prepare DNase reaction mixture: in a sterile microcentrifuge tube mix **235 µL Reaction Buffer for rDNase** and **25 µL reconstituted rDNase** (see section 3) per NucleoSpin® RNA Midi Column. Mix thoroughly but gently.

+ 250 µL rDNase reaction mixture

RT, 15 min

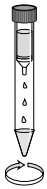
Digest with rDNase

Apply **250 µL DNase** reaction mixture directly onto the center of the silica membrane. Incubate at **room temperature** for **15 min**.

8 Wash and dry silica membrane

1st wash

Add **2.6 mL Buffer RAW2** to the NucleoSpin® RNA Midi Column. Incubate at room temperature for 2 min. Centrifuge for **3 min** at **4,500 x *g***. Discard flow-through and place the column back into the Collection Tube.



+ 2.6 mL RAW2

4,500 x *g*, 3 min

Buffer RAW2 will inactivate the rDNase.

2nd wash

Add **2.6 mL Buffer RA3** to the NucleoSpin® RNA Midi Column. Centrifuge for **3 min** at **4,500 x *g***.

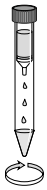
+ 2.6 mL RA3

4,500 x *g*, 3 min

The flow-through has not to be discarded in this step. Leave the NucleoSpin® RNA Midi Column in the Collection Tube.

3rd wash

Add **2.6 mL Buffer RA3** to the NucleoSpin® RNA Midi Column. Centrifuge for 5 min at **4,500 x *g*** to dry the membrane completely. Place the column into a fresh Collection Tube (15 mL, supplied).

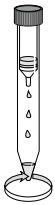


+ 2.6 mL RA3

4,500 x *g*, 5 min

9 Elute RNA

Pipette **500 µL RNase-free H_2O** (supplied) directly onto the center of the silica membrane. Incubate at **room temperature** for **2 min** and centrifuge for **3 min** at **4,500 x *g***.



+ 500 µL RNase-free H_2O

RT, 2 min

4,500 x *g*, 3 min

Reduction of elution volume will generally not result in an increased concentration of eluted nucleic acid with the NucleoSpin® RNA Midi kit (see section 2.4 for alternative elution procedures).

6.2 RNA preparation from up to 5 x 10^9 bacterial cells

Additional reagent to be supplied by user:

- Lysozyme

Before starting the preparation:

- Check that Wash Buffer RA3 and rDNase were prepared according to section 3.

1 Homogenize sample

Resuspend the **bacterial cell pellet** (Gram-negative strains) in **200 µL TE buffer** (10 mM Tris-HCl, 1 mM EDTA; pH 8) containing **1 mg/mL lysozyme** by vigorous vortexing. Incubate at **37 °C** for **10 min**.

For preparation of RNA from Gram-positive bacteria, resuspend cells in 200 µL TE containing 2 mg/mL lysozyme. It may be necessary to optimize incubation time and lysozyme concentration, depending on the bacterial strain.

2 Lyse cells

Add **1.8 mL Buffer RA1** and **18 µL ß-mercaptoethanol** to the suspension and vortex vigorously.

For appropriate sample and lysis buffer amounts see section 2.2.

Note: As alternative to ß-ME the reducing agent DTT or TCEP may be used. Use a final concentration of 10–20 mM DTT or TCEP within the Lysis Buffer RA1.

3 Filtrate lysate

Reduce viscosity and turbidity of the solution by filtration through **NucleoSpin® Filters Midi**. Apply the lysate to a NucleoSpin® Filter Midi placed in a Collection Tube, and centrifuge for **10 min** at **4,500 x *g***.

In case of visible pellet formation (depending on sample amount and nature) transfer supernatant without any formed pellet to a new 15 mL centrifuge tube (not supplied).

Proceed with step 4 of the NucleoSpin® RNA Midi standard protocol (section 6.1).

6.3 RNA preparation from up to 3 x 10^8 yeast cells

Additional reagents and components to be supplied by user:

- Reducing agent (ß-mercaptoethanol, or DTT (dithiothreithol), or TCEP (BisTris (Bis-(2-hydroxyethyl)-imino-tris(hydroxymethyl)-methane))
- Sorbitol and lyticase (or zymolase) for homogenization by enzymatic digestion or a swing-mill and glass beads for homogenization by mechanical disruption

Before starting the preparation:

- Check that Wash Buffer RA3 and rDNase were prepared according to section 3.

1 Homogenize sample

Two alternative protocols are given for homogenization of yeast cells. Users may choose between an enzymatic digestion (A) **or** mechanical homogenization (B), depending on laboratory equipment and personal preference. Homogenization by enzymatic digest is only recommended for fresh harvested cells, homogenization by mechanical disruption may also be performed with yeast cell pellets, stored at -70 °C for several months.

Note: Due to the much higher concentration of genome equivalents in a nucleic acid preparation of yeasts compared with cultured cells or tissue material, it may be necessary to use a lower quantity of cells for the preparation.

A) Homogenization by enzymatic digest

Harvest an appropriate amount of cells from **YPD culture (5,000 x *g*; 10 min)**. Resuspend pellet in an appropriate amount of fresh prepared sorbitol / lyticase buffer (50–100 U lyticase or zymolase in 1 M sorbitol / 100 mM EDTA) and incubate at **30 °C** for 30 min. Pellet the resulting spheroplasts by centrifugation (**1,000 x *g***; **10 min**).

Carefully discard supernatant.

It may be necessary to optimize incubation time and lyticase / zymolase concentration, depending on the yeast strain.

Continue with step 2.

OR

B) Homogenization by mechanical disruption

Harvest an appropriate amount of cells from **YPD culture** (**5,000 x *g***; **10 min**) and wash with ice-cold water. Resuspend the cell pellet in a mixture of 3.6 mL Buffer RA1 and 36 µL ß-mercaptoethanol.

Add glass beads (e.g., 300 mg glass beads, 425–600 µm, Sigma-Aldrich #68772).

Shake samples in a swing-mill at 30 Hz for **15 min**.

Continue with step 3.

Note: As alternative to ß-ME the reducing agent DTT or TCEP may be used. Use a final concentration of 10–20 mM DTT or TCEP within the Lysis Buffer RA1.

2 Lyse cells

Add **3.6 mL Buffer RA1** and **36 µL ß-mercaptoethanol** and vortex vigorously to lyse spheroplasts.

For appropriate sample and lysis buffer amounts see section *2.2.*

Note: As alternative to ß-ME the reducing agent DTT or TCEP may be used. Use a final concentration of 10–20 mM DTT or TCEP within the Lysis Buffer RA1.

3 Filtrate lysate

Reduce viscosity and turbidity of the solution by filtration through **NucleoSpin® Filter Midi**. Place NucleoSpin® Filter Midi placed in Collection Tubes and centrifuge for **10 min** at **4,500 x** *g*.

In case of visible pellet formation (depending on sample amount and nature) transfer supernatant without any formed pellet to a new 15 mL centrifuge tube (not supplied).

4 Adjust RNA binding conditions

Discard the NucleoSpin® Filter Midi and add **3.6 mL 70 % ethanol** to the lysate in the Collection Tube and mix by vortexing.

Proceed with step 5 of the NucleoSpin® RNA Midi standard protocol (section 6.1).

6.4 Clean-up of RNA from reaction mixtures

Before starting the preparation:

- Check that Wash Buffer RA3 was prepared according to section 3.

1 Prepare sample

Fill up RNA samples smaller than 500 µL with RNase-free H_2O to 500 µL.

2 Prepare lysis-binding buffer premix

Prepare a Buffer RA1 - ethanol premix with ratio 1 : 1.

For **each 500 µL RNA sample**, mix **1500 µL Buffer RA1** and **1500 µL ethanol (96–100 %)**.

If multiple samples are processed, the preparation of a master-premix is recommended (e.g., 2 mL RA1 + 2 mL 98 % ethanol for approximately 6 NucleoSpin® RNA preparations).

3 Filtrate lysate

Not necessary!

4 Adjust RNA binding conditions

To **500 µL of RNA sample, add 3000 µL (6 volumes) of Buffer RA1-ethanol premix**. Mix sample with premix by vortexing.

Maximal loading capacity of NucleoSpin® RNA Midi Columns is 4000 µL. Repeat the procedure if larger volumes are to be processed.

After addition of ethanol a stringy precipitate may become visible which will not affect the RNA isolation. Be sure to mix thoroughly and apply sample as homogenious solution onto the column. For binding capacity of the columns, see Table 1.

Proceed with step 5, 8, and 9 of NucleoSpin® RNA Midi standard protocol (section 6.1). Steps 6 and 7 of the respective protocols may be omitted in this case.

As alternative products for RNA clean-up, NucleoSpin® RNA Clean-up and NucleoSpin® RNA Clean-up XS are recommended (see ordering information).

7 NucleoSpin® RNA / NucleoSpin® RNA Midi protocols

7.1 RNA preparation from RNA*later*® treated samples

Before starting the preparation:

- Check that Wash Buffer RA3 and rDNase were prepared according to section 3.

1	**Prepare sample** Remove RNA*later*® solution. Cut an appropriate amount of tissue.
2	**Lyse cells** Add **350 µL** (NucleoSpin® RNA) / **1.8 mL** (NucleoSpin® RNA Midi) **Buffer RA1** and **3.5 µL** (NucleoSpin® RNA) / **18 µL** (NucleoSpin® RNA Midi) **ß-mercaptoethanol** to the sample. Disrupt the sample material by using, for example, rotor-stator homogenizers (for homogenization methods see section 2.3).
	Proceed with step 3 (filtrate lysate) of the NucleoSpin® RNA standard protocol (section 5.1) or NucleoSpin® RNA Midi standard protocol (section 6.1).

7.2 rDNase digestion in solution

The on-column rDNase digestion in the standard protocol is already very efficient and thus resulting in minimal residual DNA. This DNA will not be detectable in most downstream applications. Despite this, there are still certain applications which require even lower contents of residual DNA. However, removal of DNA to a completely undetectable level is challenging and the efficiency of an on-column DNA digestion is sometimes not sufficient for downstream applications requiring lowest residual content of DNA.

A typical example for such a demanding application is an RT-PCR reaction in which the primer molecules do not differentiate between cDNA (derived from RNA) and contaminating genomic DNA. Especially, if

- high copy number targets are analyzed (e.g., multi gene family, mitochondrial, plastidal or plasmid targets (from transfections))
- the target gene is of a very low expression level
- the amplicon is relatively small (< 200 bp).

DNA digestion in solution can efficiently destroy contaminating DNA. However, stringent RNase control and subsequent repurification of the RNA (in order to remove buffer, salts, DNase and digested DNA) are usually required.

The high quality, recombinant, RNase-free DNase (rDNase) in the NucleoSpin® RNA kits facilitates such a digestion in solution in order to remove even traces of contaminating DNA.

A	**Digest DNA (Reaction setup)** Add **6 µL Reaction Buffer for rDNase** and **0.6 µL rDNase** to **60 µL eluted RNA**. (Alternatively premix 100 µL Reaction Buffer for rDNase and 10 µL rDNase and add 1/10 volume to one volume of RNA eluate). Gently swirl the tube in order to mix the solution. Spin down gently (approx. 1 s at 1,000 x *g*) to collect every droplet of the solution at the bottom of the tube.
B	**Incubate sample** Incubate for **10 min** at **37 °C**.

C **Repurify RNA**

Repurify RNA with a suitable RNA cleanup procedure, for example by use of the NucleoSpin® RNA Clean-up, NucleoSpin® RNA Clean-up XS kits (see ordering information), or by ethanol precipitation.

Ethanol precipitation, exemplary:

Add **0.1 volume** of **3 M sodium acetate, pH 5.2** and **2.5 volumes** of **96–100 % ethanol** to **one volume of sample**. Mix thoroughly.

Incubate **several minutes** to **several hours** at **-20 °C** or **4 °C**.

Note: Choose long incubation times if the sample contains low RNA concentration. Short incubation times are sufficient if the sample contains high RNA concentration.

Centrifuge for **10 min** at **maximum speed**.

Wash RNA pellet with **70 % ethanol**.

Dry RNA pellets and resuspend RNA in RNase-free H_2O.

8 Appendix

8.1 Troubleshooting

Problem	Possible cause and suggestions
RNA is degraded / no RNA obtained	*RNase contamination* • Create an RNase free working environment. Wear gloves during all steps of the procedure. Change gloves frequently. Use of sterile, disposable polypropylene tubes is recommended. Keep tubes closed whenever possible during the preparation. Glassware should be oven-baked for at least 2 hours at 250 °C before use.
Poor RNA quality or yield	*Reagents not applied or restored properly* • Reagents not properly restored. Add the indicated volume of RNase-free H_2O to rDNase vial and 96 % ethanol to Buffer RA3 Concentrate and mix. Reconstitute and store lyophilized rDNase according to instructions given in section 3. • Sample and reagents have not been mixed completely. Always vortex vigorously after each reagent has been added. • No ethanol has been added after lysis. Binding of RNA to the silica membrane is only effective in the presence of ethanol. *Kit storage* • Reconstitute and store lyophilized rDNase according to instructions given in section 3. • Store other kit components at room temperature. Storage at low temperatures may cause salt precipitation. • Keep bottles tightly closed in order to prevent evaporation or contamination. *Ionic strength and pH influence A_{260} absorption as well as ratio A_{260}/A_{280}* • For absorption measurement, use 5 mM Tris pH 8.5 as diluent. Please see also: - Manchester, K L. 1995. Value of A_{260}/A_{280} ratios for measurement of purity of nucleic acids. Biotechniques 19, 208–209. - Wilfinger, W W, Mackey, K and Chomczyski, P. 1997. Effect of pH and ionic strength on the spectrophotometric assessment of nucleic acid purity. Biotechniques 22, 474–481.

Problem	Possible cause and suggestions
Poor RNA quality or yield *(continued)*	*Sample material* • Sample material not stored properly. Whenever possible, use fresh material. If this is not possible, flash freeze the samples in liquid N_2. Samples should always be kept at -70 °C. Never allow tissues to thaw before addition of Buffer RA1. Perform disruption of samples in liquid N_2. • Insufficient disruption and / or homogenization of starting material. Ensure thorough sample disruption and use NucleoSpin® Filters / Filters Midi for easy homogenization of disrupted starting material.
Low A_{260} / A_{230} ratio	*Carry-over of guanidinium thiocyanate* • Carefully load the lysate to the NucleoSpin® RNA Column and try to avoid a contamination of the upper part of the column and the column lid. • Make sure that a sufficient amount / concentration of RNA is used for quantification so that the A_{230} value is significantly higher than the background level.
Clogged NucleoSpin® Column / Poor RNA quality or yield	Sample material • Too much starting material used. Overloading may lead to decreased overall yield. Reduce amount of sample material or use larger volume of Buffer RA1. • Insufficient disruption and / or homogenization of starting material. Ensure thorough sample disruption and use NucleoSpin® Filters / Filters L for easy homogenization of disrupted starting material.
Contamination of RNA with genomic DNA	*rDNase not active* • Reconstitute and store lyophilized rDNase according to instructions given in section 3. *DNase solution not properly applied* • Pipette rDNase solution directly onto the center of the silica membrane. *Too much cell material used* • Reduce quantity of cells or tissue used.

Problem	Possible cause and suggestions
Contamination of RNA with genomic DNA *(continued)*	*DNA detection system too sensitive* The amount of DNA contamination is effectively reduced during the on-column digestion with rDNase. However, it can not be guaranteed that the purified RNA is 100 % free of DNA, therefore in very sensitive applications it might still be possible to detect DNA. The NucleoSpin® RNA / Plant system is checked by the following procedure: One million HeLa cells are subjected to RNA isolation according to the protocol. RNA eluate is used as template for PCR detection of a 1 kb fragment in a 30 cycle reaction. Generally, no PCR product is obtained while skipping the DNase digest usually leads to positive PCR results. The probability of DNA detection with PCR increases with: - the number of DNA copies per preparation: single copy target < plastidial / mitochondrial target < plasmid transfected into cells - decreasing of PCR amplicon size. • Use larger PCR targets (e.g., > 500 bp) or intron spanning primers if possible. • Use support protocol 7.2 for subsequent rDNase digestion in solution.
Suboptimal performance of RNA in downstream experiments	*Carry-over of ethanol or salt* • Do not let the flow-through touch the column outlet after the second Buffer RA3 wash. Be sure to centrifuge at the corresponding speed for the respective time in order to remove ethanolic Buffer RA3 completely. • Check if Buffer RA3 has been equilibrated to room temperature before use. Washing at lower temperatures lowers efficiency of salt removal by Buffer RA3. *Store isolated RNA properly* • Eluted RNA should always be kept on ice for optimal stability since trace contaminations of omnipresent RNases (general lab ware, fingerprints, dust) will degrade the isolated RNA. For short term storage freeze at -20 °C, for long term storage freeze at -70 °C.

8.2 Ordering information

Product	REF	Pack of
NucleoSpin® RNA	740955.10 / .50 / .250	10 / 50 / 250
NucleoSpin® RNA Midi	740962.20	20
NucleoSpin® miRNA	740971.10 / .50 / .250	10 / 50 / 250 preps
NucleoSpin® RNA / Protein	740933.10 / .50 / .250	10 / 50 / 250
NucleoSpin® TriPrep	740966.10 / .50 / .250	10 / 50 / 250
NucleoSpin® totalRNA FFPE	740982.10 / .50 / .250	10 / 50 / 250
NucleoSpin® RNA Plant	740949.10 / .50 / .250	10 / 50 / 250
NucleoSpin® RNA Blood	740200.10 / .50	10 / 50
NucleoSpin® RNA Blood Midi	740210.20	20
NucleoSpin® 8 RNA Blood	740220 / .5	12 x 8 / 60 x 8
NucleoSpin® 96 RNA Blood	740225.2 / .4	2 x 96 / 4 x 96
NucleoSpin® RNA Clean-up	740948.10 / .50 / .250	10 / 50 / 250
NucleoSpin® RNA XS	740902.10 / .50 / .250	10 / 50 / 250
NucleoSpin® RNA Clean-up XS	740903.10 / .50 / .250	10 / 50 / 250
NucleoSpin® totalRNA FFPE XS	740969.10 / .50 / .250	10 / 50 / 250
NucleoSpin® RNA / DNA Buffer Set	740944	Suitable for 100 preps
Buffer RA1	740961	60 mL
Buffer RA1	740961.500	500 mL
rDNase Set	740963	1 set
TCEP	740395.107	107 mg
NucleoSpin® Filters	740606	50
Collection Tubes (2 mL)	740600	1000

Visit ***www.mn-net.com*** for more detailed product information.

8.3 Product use restriction/warranty

NucleoSpin® RNA kit components are intended, developed, designed, and sold FOR RESEARCH PURPOSES ONLY, except, however, any other function of the product being expressly described in original MACHEREY-NAGEL product leaflets.

MACHEREY-NAGEL products are intended for GENERAL LABORATORY USE ONLY! MACHEREY-NAGEL products are suited for QUALIFIED PERSONNEL ONLY! MACHEREY-NAGEL products shall in any event only be used wearing adequate PROTECTIVE CLOTHING. For detailed information please refer to the respective Material Safety Data Sheet of the product! MACHEREY-NAGEL products shall exclusively be used in an ADEQUATE TEST ENVIRONMENT. MACHEREY-NAGEL does not assume any responsibility for damages due to improper application of our products in other fields of application. Application on the human body is STRICTLY FORBIDDEN. The respective user is liable for any and all damages resulting from such application.

DNA/RNA/PROTEIN purification products of MACHEREY-NAGEL are suitable for IN-VITRO-USES ONLY!

ONLY MACHEREY-NAGEL products specially labeled as IVD are also suitable for IN-VITRO-diagnostic use. Please pay attention to the package of the product. IN-VITRO-diagnostic products are expressly marked as IVD on the packaging.

IF THERE IS NO IVD SIGN, THE PRODUCT SHALL NOT BE SUITABLE FOR IN-VITRO-DIAGNOSTIC USE!

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Please contact:
MACHEREY-NAGEL GmbH & Co. KG
Tel.: +49 24 21 969-270
tech-bio@mn-net.com

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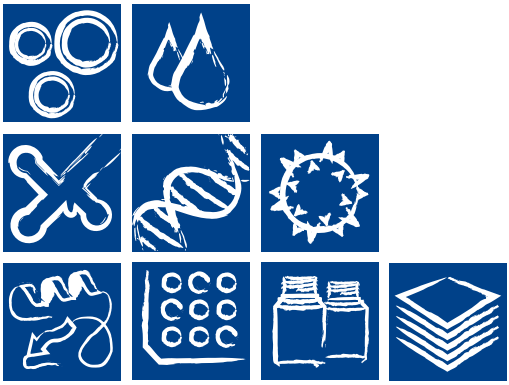

MACHEREY-NAGEL

MACHEREY-NAGEL GmbH & Co. KG · Neumann-Neander-Str. 6–8 · 52355 Düren · Germany

Germany and international:
Tel.: +49 24 21 969-0
E-mail: info@mn-net.com

Switzerland:
MACHEREY-NAGEL AG
Tel.: +41 62 388 55 00
E-mail: sales-ch@mn-net.com

France:
MACHEREY-NAGEL EURL
Tel.: +33 388 68 22 68
E-mail: sales-fr@mn-net.com

USA:
MACHEREY-NAGEL Inc.
Tel.: +1 484 821 0984
E-mail: sales-us@mn-net.com

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